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ISOLATION OF ASCARIS HAEMOGLOBINS

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SUMMARY

1. The two haemoglobins of Ascaris have been studied in an effort to purify them, and to determine their approximate molecular weights and general spectral properties.

2. It has not been found possible to obtain either haemoglobin free from contaminating protein except during ultracentrifugation.

3. The isoelectric points of the haemoglobins are about 5.0 and 6.7.

4. The molecular weights are about 14000 and 280000. These are uncommon values for haemoglobins and are apparently the first reported for nematode worms.

5. The general spectral characters of both haemoglobins conform to those of other haemoglobins. However, even allowing for contamination, the protein absorption seems to be unusually high. It has not been possible to determine the reason for this.

6. The properties of both haemoglobins differ from those of mammalian haemoglobin in every respect examined, and they are therefore specific proteins produced by the worm itself.

INTRODUCTION

The work described in this paper was complementary to a study of the metabolism of the haemoglobins of *Ascaris lumbricoides* of the pig, which is discussed elsewhere[1]. The object of the experiments was to obtain further purification of the haemoglobins and to establish their molecular weights and general spectral properties for comparison with known haemoglobins. Some general information and spectral data have been given by DAVENPORT[2] and by TREIBS, MENDHEIM AND LORENZ[3]. Further data on physico-chemical properties are given in an accompanying paper[4].

MATERIALS AND METHODS

Spectrophotometry was generally carried out with the Beckman DU spectrophotometer but spectra of a number of haemoglobin derivatives were obtained with a Beckman DK-2 recording spectrophotometer over the range 350–700 mμ. Extinction coefficients are based on the assumption that the maximum extinction of the met

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cyanide derivative of each haemoglobin in the visible is 11.5, the value which is taken as a general standard[5].

Electrophoresis was carried out at approx. 23° on Whatman 3MM paper immersed in CCl_4 or chlorobenzene as heat dispersant, using deep Perspex electrode vessels of 2 l capacity fitted with platinum electrodes. Mains voltage was applied directly after rectification, and the current passing was measured with a milliammeter.

Ultracentrifuge analyses were carried out with the Spinco Model E.

Column chromatography was performed using Whatman DEAE-cellulose powder and Sephadex G-75.

Solutions of perienteric fluid haemoglobin were obtained by collecting body fluid from the worms. Solutions of the body-wall haemoglobin were obtained by extracting the bodies of eviscerated worms (previously stored at −5°) with a little water or buffer for 1–2 h in the refrigerator. Both haemoglobins were concentrated and partly purified in the following way. The crude solution was first centrifuged and dialysed against 70% satd. neutralised ammonium sulphate. The precipitate which formed was removed by centrifugation. The supernatant then contained the bulk of the haemoglobin which was precipitated by increasing the ammonium sulphate to 85% saturation. The precipitate was collected by centrifugation and was redissolved in a small amount of buffer or distilled water.

EXPERIMENTAL

Further purification

Considerable effort was devoted to attempts to purify both haemoglobins, using electrophoresis and column chromatography as preparative techniques, and taking the 280:400-mμ ratio as an index of purity in the usual way. For known haemoglobins this ratio is 0.3 or less, but it has not so far been possible to obtain a preparation of Ascaris haemoglobin in which the ratio was less than 1. The possible reasons for this are complex and will be referred to again farther on. At the present time the purest preparations have been obtained by chromatography.

Electrophoresis: Paper electrophoresis of both haemoglobins was carried out in various buffers of about I 0.3 over the pH range 5–9. Horse haemoglobin was used as a marker. Each Ascaris haemoglobin ran as a single component, though the presence of other colourless components could be shown by staining for protein. Haemoglobin separated in this way was eluted from the paper. The 280:400-mμ ratio of such preparations was still more than 1. The results of electrophoresis at the various pH values suggest that the perienteric-fluid haemoglobin has an isoelectric point in the region of 5.0, while that of the body-wall haemoglobin is in the region of 6.7.

Column chromatography: The powder form of Whatman DEAE-cellulose was washed on a Buchner funnel with distilled water until the filtrate was free of material absorbing at 280 mμ. 10 g of the powder were then suspended in 150 ml of 0.1 M Tris–HCl buffer (pH 7.2). The powder was stirred in the buffer for approx. 15 min, and the pH was then adjusted to 7.2 with 0.1 M HCl. This procedure was repeated until the pH remained constant at 7.2. The cellulose was then washed with distilled water until free of buffer.

A column, 20 cm in height and 1 cm in dia., was prepared with the buffered cellulose at room temperature. 100 ml of 0.05 M Tris–HCl buffer (pH 7.2) were then

allowed to pass through the column. 1.5 ml of a methaemoglobin solution, previously dialysed against the same buffer containing 0.001 M cyanide, were then allowed to pass through the column. After the protein had entered the column, the walls of the chromatographic tube were washed twice with 2-ml portions of the same buffer. The protein was then displaced from the column by gradient elution. A reservoir containing 100 ml of the buffer plus cyanide was connected to the column so that about 20 ml of buffer were maintained above the column. The gradient was provided by connecting a second reservoir, containing buffer–cyanide with 0.5 M NaCl, to the first. The eluent was collected with a drop-count fraction collector and each tube was analysed spectrophotometrically. After 300 ml had been collected, the elutant was changed to 1 M NH_4Cl and a last fraction was collected.

(*a*) *Perienteric-fluid haemoglobin*: The haemoglobin was separated into 4 haemoprotein fractions with the technique described. Fraction 1 contained a mixture of proteins which were not retained by the ion-exchange cellulose; Fraction 2 was a haemoglobin fraction whose concentration appeared to vary from one preparation to another; Fraction 3 was the major fraction; and Fraction 4 was eluted with NH_4Cl. The first 3 fractions contained approx. 2%, 4%, and 82%, respectively, of the haemoglobin eluted with the NaCl gradient, the remainder being present in the long tail which follows Fraction 3.

Fraction 3 was precipitated with ammonium sulphate and rechromatographed after dialysis against the buffer. The gradient elution now gave a single component whose position in the elution pattern was identical to that obtained in the original chromatogram. However, a portion of the haemoglobin was not eluted by the gradient procedure. It undoubtedly represented denatured protein and could be eluted with NH_4Cl. This suggests that Fraction 4 represents denatured haemoglobin.

The 280:400-mμ ratio of Fraction 3 was 1 or a little above. Attempts to improve the ratio by chromatography at other pH's (using various buffers) were not successful. Although a varying amount of separation was possible, the ratio was usually not less than 1.1.

Samples of the haemoglobin were also run through columns of Sephadex G-75, in phosphate buffer (pH 6.4). The haemoglobin was not retained by the gel, and again, although there was some separation from colourless protein, the protein:haem ratio was still above 1. It was judged that in view of the dilution resulting from the use of Sephadex columns, this technique did not offer any advantages in purifying the crude body fluid, although it did bring the 280:400-mμ ratio of this from about 5 to 2.

(*b*) *Body-wall haemoglobin*: It has not yet been possible to extend the chromatographic experiments with DEAE-cellulose to the body-wall haemoglobin. When it was run through Sephadex G-75 in the same way as the perienteric-fluid haemoglobin, there was retention of the haemoprotein, but the 280:400-mμ ratio of the collected fraction was much above 1.

Ultracentrifugation

Perienteric-fluid haemoglobin: Samples of this haemoglobin fractionated with ammonium sulphate and DEAE-cellulose chromatography (Fraction 3 lyophilised) were examined by Dr. R. MARKHAM of the Virus Research Unit, University of Cambridge. The haemoglobin was equilibrated against buffer containing 0.1 M NaCl

before carrying out the run. The sedimentation behaviour was followed at 128000 × *g* using Schlieren optics, with a protein concentration of about 3 mg/ml.

The haemoglobin peak appeared to sediment homogeneously with a coefficient of 11.6 S. In the purest sample, Fraction 3, from DEAE-cellulose, there were also a small fraction with a coefficient of about 18 S, and a slow fraction with a coefficient of 2 S. The slow fraction was calculated to be present in about 70 % of the concentration of the haemoglobin by taking the areas under the peaks of the photographic trace. A rough value of the diffusion constant was also calculated from the rate of spreading of the haemoglobin peak using the method of moments. The rate was found to be constant and gave an apparent coefficient of 6.9 F.

Body-wall haemoglobin: Body-wall haemoglobin fractionated with ammonium sulphate was run under the same conditions, but at 260000 × *g*. These runs were carried out in the Biochemistry Department, University of Cambridge, by Mr. B. E. BOON. Two colourless components with approximate coefficients of 10 and 5 S were observed, but the coloured material appeared to sediment homogeneously with a coefficient of 1.55 S. The apparent diffusion coefficient was 16 F, and the rate of spreading appeared to be uniform.

Spectroscopic observations

Spectral observations were carried out on the material prepared for general studies. The resulting data (shown in Table I) are preliminary and are liable to revision

TABLE I

ABSORPTION MAXIMA AND EXTINCTION COEFFICIENTS (PER MMOLE OF HAEMATIN) OF ASCARIS HAEMOGLOBINS

Values in parentheses are derived from DAVENPORT[2] (D) and TREIBS *et al.*[3] (T).

Derivative	*Perienteric-fluid Hb*		*Body-wall Hb*	
	λ_{max} (mμ)	ε_{mM}	λ_{max} (mμ)	ε_{mM}
Oxidised				
Acid	490/500 (D 500) (T 505)	10.7	490 (D 500)	11.8
	630 (D 630) (T 635)	3.8	630 (D 630)	4.3
Alkaline	(ↄ 537)		540 (D 537)	10.2
	(D 565)		580 (D 565)	7.8
Cyanide	540 (D 544)	[11.5] (D 11.2)	540	[11.5]
Fluoride	540	8.4		
	600	6.6	605	7.1
Azide	540	9.6	540	9.6
Reduced				
Deoxygenated	551 (D 555) (T 553.5)	10.8 (D 11.7)	551 (D 555)*	11.5 (D 11.7)*
Carboxy	414			
	540 (T 544)	10.6	(D 539)*	(D 12.3)*
	560 (T 572)	10.5	(D 566)*	(D 12.7)*
Oxy	407	161	410	98.3
	540 (D 542) (T 543)	12.3	541 (D 543)*	12.4 (D 13.5)*
	578 (D 578) (T 579)	9.5	579 (D 580)*	10.6 (D 11.7)*

* Values obtained from Fig. 1 of DAVENPORT.

when the pure haemoglobins are isolated. Values for the protein band at about 280 mμ cannot be given because of the presence of large amounts of colourless protein. This does not affect the values quoted here since the cyanide method measures haematin concentration directly and is independent of the presence of extraneous protein, or of changes in the protein portion of the haemoglobin.

DISCUSSION

The results of the work described in the experimental section have in all cases corroborated the conclusion of SMITH AND LEE[1] that both Ascaris haemoglobins are quite distinct from the haemoglobin of the host. They must therefore be considered as specific native proteins synthesised by the worm itself.

Purification

Our technique for preliminary fractionation with ammonium sulphate was based on the results of two salting-out experiments in which precipitates were collected as the concentration of ammonium sulphate was increased. It appeared that maximum precipitation of the haemoglobins occurred at about 73%, and that by collecting the fraction precipitating between 70 and 85% one would achieve maximum yield with minimum contamination. Even by isolating the 73% precipitate, however, the 280:400-mμ ratio could not be reduced below about 1.2. On the other hand, outside the range 70–85% the proportion of extraneous protein increases appreciably, and we are unable to account for DAVENPORT'S recommendation[2] that all material precipitating between 53 and 73% satn. be collected. Unfortunately he does not give any spectroscopic data for the 280-mμ band, though he does state that the collected fraction contained 12% haemoglobin (from haematin concentration and dry weight). In view of the uncertainties about the protein absorption of the haemoglobin it is difficult to known how to interpret this, but the absorption of the extraneous protein would have to be very low to give a 280:400-mμ ratio of 1 or less when present in 7-fold excess. There seems to be no justification for collecting material precipitating in the range 53–70% satn. when this contains little if any of the haemoglobin.

Ultracentrifuge analysis of Ascaris oxyhaemoglobin having a 280:400-mμ ratio of 1 showed that at least 2 components were present, one of which was colourless. The high protein to haematin ratio is therefore due in the first instance to the presence of contaminating protein. Calculation of the relative proportions of haemoglobin and contaminating protein suggested that the haemoglobin itself might also have an unusually high protein absorption. This could be due to a larger than usual number of amino acid residues per haem, or to the presence of unusually large amounts of the aromatic amino acids which are responsible for the protein absorption. SCHELER AND SCHNEIDERAT[6] have reported that the haemoglobin of Tubifex has only 60 haems per molecule of molecular weight $3 \cdot 10^6$, *i.e.* 1 haem per 50000 equiv. We have been unable to find any other similar report in the literature. The question deserves further examination both for its own intrinsic interest, and also as bearing on the relationship between haemoglobins and catalases, which also have only 1 haem per 50–70000 equiv. In the case of the Ascaris haemoglobins a similar proportion of haem to protein would increase the proportion of protein absorption by 3–4 times, thus providing an immediate explanation for the observed 280:400-mμ ratio. However it would be un-

warranted to draw such a conclusion at this stage, if only because of the possible differences between the annelid, Tubifex, and the nematode, Ascaris.

Molecular weights

The sedimentation studies were quite surprising in themselves. It appears that Ascaris contains haemoglobins which, apart from their other unusual properties, have very uncommon molecular weights. Although the partial specific volumes are not known, the diffusion coefficients are known accurately enough to show that both haemoglobins have the same sort of sedimentation behaviour as other haemoglobins, *i.e.* they must have the same general external shape. If we assume for each a partial specific volume of 0.74 and a frictional coefficient of 1.25 (values characteristic of other haemoglobins), then the observed sedimentation coefficients of 1.55 and 11.6 S correspond to molecular weights of 14000 and 280000, with diffusion coefficients of 10.6 and 3.9 F. The apparent diffusion coefficients are only 1.5 and 2 times greater, which is very reasonable agreement for the method used to calculate them.

The body-wall Ascaris haemoglobin is one of the two smallest haemoglobins known, the other being that of Paramecium[7], which has a molecular weight of 13400. The perienteric-fluid haemoglobin is the first commonly occurring haemoglobin of about 300000 molecular weight. SVEDBERG AND HEDENIUS[8] stated that a component of 12.5 S was observed occasionally in aged reptilian blood. A later statement[9] attributed such a component to amphibian blood only. It seems possible that any such component is actually an artifact of preparation.

It was also quite unexpected that this Ascaris haemoglobin should have as low a molecular weight as 300000, since it was considered a general rule[10] that extracellular haemoglobins have molecular weights greater than 400000. No worm was previously known in which the molecular weight of an extra-cellular haemoglobin was less than about $3 \cdot 10^6$. Surprisingly, however, we find that this applies to annelids only, and we have been unable to trace any previous value for the molecular weight of a nematode haemoglobin. It will therefore be of considerable interest to determine whether haemoglobins of other nematodes differ from Ascaris in this respect.

NOTE ADDED IN PROOF

ANTONINI, ROSSI-FANELLI AND CAPUTO[11] have reported that *Spirographis chlorocruorin* has 80 haems per molecule of molecular weight $2.8 \cdot 10^6$, *i.e.* 1 haem per 35000 equiv. Such pigments may be regarded as annelid haemoglobins with a modified haem group, so that this observation reinforces that of SCHELER AND SCHNEIDERAT[6].

Since this manuscript was submitted, HAMADA *et al.*[12] have begun to publish a series of papers on Ascaris body-wall haemoglobin. Their observations agree with ours in some respects, but in others differ markedly. The major discrepancy is that they have resolved the pigment into two components, one of which has a sedimentation coefficient of 3.2 S. We have repeated the extraction using fresh material and extracting for 48 h as the Japanese workers did. The results were not affected by this change and we have never observed a haemoprotein with such a sedimentation coefficient. Since some of their spectral data conflicts with Table I in such a way as to suggest partial denaturation of their materials, the possibility appears to exist that their final preparation no longer consists of native haemoglobin.

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SOME ASPECTS OF THE COMBINATION OF ASCARIS HAEMOGLOBINS WITH OXYGEN AND CARBON MONOXIDE

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SUMMARY

1. Previous studies on the kinetics of the deoxygenation of the two Ascaris haemoglobins have been confirmed and extended. It has been shown that biochemical reducing systems produce the same results as sodium dithionite, so that no artifacts are introduced by using the latter under the conditions specified.

2. The deoxygenation velocity of the body-wall haemoglobin has a Q_{10} of about 3 and changes markedly with pH, whereas that of the perienteric-fluid haemoglobin has a Q_{10} of 5 and is little affected by change in pH. The reaction of the body-wall haemoglobin is about 17 times as fast as that of the perienteric fluid at 38°.

3. Further data are given to support the previous observation that oxyhaemoglobins in which the β band is more intense than the α band always seem to have a high affinity for O_2. Both the Ascaris haemoglobins are of this type.

4. Data obtained in this study have made it possible to estimate the value of the partition coefficient for both haemoglobins. It is shown that in both cases this value is not more than 0.1, and is probably nearer to 0.01. These values are much lower than any previously reported for haemoglobins.

INTRODUCTION

The properties and function of the body-wall and perienteric-fluid haemoglobins of pig *Ascaris lumbricoides* have recently been extensively investigated[1,2]. This paper

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